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Art Unit: **1634**

For: **Process for Preparing an Anti-Oxidant**

Examiner: **Kallis, Russell**

DECLARATION UNDER RULE 37 CFR 1.132

I, Anders Boegh Jensen of Poalis A/S Bülowsvej 25 DO SOLEMNLY and **SINCERELY DECLARE** as follows:

1. I hold a B.Sc. degree in Biochemistry, Ph.D. in biochemistry from the University of Copenhagen, Denmark.

2. I have worked in the Food Industry since January 2003

3. Glucan lyases (EC 4.2.2.13) are enzymes, which produce 1,5-anhydro-D-fructose (anhydrofructose) from starch and related oligomers and polymers. In plants, starch biosynthesis takes place exclusively in plastids that are the sole location of starch synthases and starch branching enzymes (Preiss 1997, Manipulation of starch synthesis. In A Molecular Approach to Primary Metabolism in Plants (Quick, W.P. and Foyer, C.H., eds). London: Taylor and Francis, pp. 81-103).

The following experiment was undertaken to show that by following the method of International Patent Application No. PCT/IB98/00708 – Publication number WO 98/50532 (from which US SN 09/423,126 derives) together with the skilled person's common general knowledge before May 1997 - anhydrofructose could be produced *in*

situ in potato plants by transforming potato plants with a nucleotide sequence encoding a glucan lyase enzyme.

Plasmid construction

To target glucan lyase protein accumulation to potato plastids a DNA fragment with gene bank accession number Y18737 corresponding to the protein sequence of Seq ID No 1 from WO98/50532, called GLq1, was inserted into the cloning vector pBluescript KS^+ from Stratagene as described International Patent Application Number WO95/10618A3.

From this DNA fragment, the first 49 amino acids were exchanged with a DNA fragment containing the first 55 amino acids of the ribulose bisphosphate carboxylase small chain 1b precursor, which encodes an efficient plastid transit peptide by overlapping PCR. First, the plastid transit peptide of ribulose bisphosphate carboxylase small chain 1b precursor (Dedonder et al. (1993), Plant Physiol. 101: 801-8) was amplified by PCR primers 5'-TGCTCTAGAGAACAATGGCTTCCTCTATGC-3' and 5'-GTTTGTCGGACAATGCGGTCATGCAGTTAACTCTTCCGCC-3'. Secondly, a 413 bp DNA fragment of the 5' end of GLq1 was amplified by PCR primers 5'-TGCTCTAGACAACAATGTTTTCAACCCTTGCGTTTGTC-3' and 5'-GTATGACGTGACCTGAACCTG-3'. The Two DNA fragments was mixed together in equal amounts, heated to 95 C for 3 min and renatured. PCR amplification with primers 5'-TGCTCTAGAGAACAATGGCTTCCTCTATGC-3' and 5'-GTATGACGTGACCTGAACCTG-3'resulted in a DNA fragment where the plastid transit peptide from ribulose bisphosphate carboxylase small chain 1b precursor was fused to the 5' end of the GLq1 DNA fragment. This fragment was digested with XbaI and SmaI and reinserted into the GLq1 gene in the cloning vector pBluescript KS^+ to generate the modified GLq1 gene designated signal-GLq1.

The complete coding sequence of signal-GLq1 was digested with XbaI and BglII and inserted between the 35S promoter and the E9 terminator sequence in plasmid pCAMBIA 2300-35S-E9 digested with the same enzymes to create the final plasmid pCAMBIA 2300-35S-signal-GLq1-E9.

This plasmid was inserted into the *Agrobacterium tumefaciens* strain LBA 4404 as described in the patent.

Transformation of potatoes

Transgenic potatoes was prepared according to the description in WO98/50532.
The transgenic genotype of the regenerated shoots was verified by PCR. A leaf was excised from plants showing normal root growth on medium containing kanamycin. DNA was extracted according to Dellaporta et. Al., 1983, Plant Mol. Biol. Rep. 1, 19-21. PCR amplification using primers
5'- AGCGGATAACAATTTCACACAGGA-3' and 5'-GTATGACGTGACCTGAACCTG-3' specific for the inserted T-DNA revealed that all plants showing normal root growth on medium containing kanamycin produced a band of the right size whereas in untransformed control plants no band was detected.

In total, 24 individual transgenic plants was produced that showed resistance towards kanamycin and contained the glucan lyase gene.

Glucan lyase activity in plants

To determine whether these 24 transgenic lines expressed and accumulated a functional glucan lyase gene total protein extracts was prepared from leaves of wild type and transgenic potato plants. Approximately 200 mg of leaf tissue was ground in 0.6 ml protein extraction buffer (20 mM Tris pH 7,5, 150 mM NaCl and 1 mM EDTA) and centrifuged for 20 minutes at 15.000 rpm at 4 deg C. The supernatant was transferred to a new tube at kept at 4 deg C. The protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories) according to the recommendations of the manufacturer. Typically, 5mg/ml of protein was extracted by this procedure. To detect glucan lyase activity, 0.5 mg total protein extract was diluted with 100 mM potassium acetate pH 5.5 to 0.250 ml. Then 0.250 ml 100 mM potassium acetate pH 5.5 with 10 mg/ml glycogen was added and the reaction was incubated at 40 deg C for 60 minutes. The reaction was stopped by heating at 100 deg

C for 2 minutes before anhydrofructose was determined by 3.5 dinitrosalicylic acid under alkaline conditions as described by Yu et. in WO94/09122.

Results showed that of the 24 individual transgenic potato lines two lines did not produce anhydrofructose, seven lines produced less anhydrofructose than 50 micrograms per 0.5 mg protein extract. The resulting 15 lines produced from 165 to 524 micrograms anhydrofructose per 0.5 mg total protein extract (Table 1).

Table 1

Line No.	Trangenic as tested by PCR	Glucan Lyase activity microgram AF/500 microgram protein	Lines tested for AF *in situ*
5.1	yes	470.3	
6.1	yes	263.8	
8.1	yes	0	yes
9.1	yes	247.3	
11.1	yes	409.1	
14.1	yes	210.8	yes
14.2	yes	0	
14.3	yes	337.2	yes
11.2	yes	524	
22	yes	234.8	
22.2	yes	427.4	
23.2	yes	0	
24	yes	511.1	
21.3	yes	48.6	
23.4	yes	36.5	
17.2	yes	28	
33.1	yes	0	
27	yes	164.9	
31	yes	510.1	
29	yes	456.1	yes
6.2	yes	23.0	
6.3	yes	23	
4.4	yes	0	
31.1	yes	432.4	
wt	no	0	yes

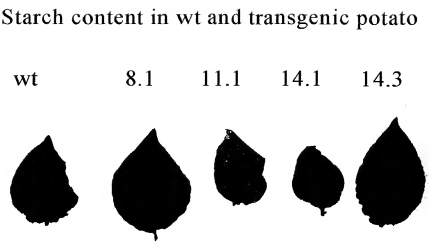
Regular visual inspection of the plants did neither reveal phenotypic alterations between the 24 transgenic lines nor when compared to wild type plants grow under the same conditions without kanmycin in the growth medium. These results demonstrates that plants can be engineered to express an active glucan lyase gene and cultivated on synthetic medium under sterile conditions with out negative effects on their growth.

Detection of anhydrofructose in transgenic plants

Since potato plants grown on synthetic LS medium only synthesize very low amounts of starch, transgenic and wild type plants (height approx. 8-10 cms) were transferred to soil and placed in a growth chamber. When growth were well established the plants were transferred to a greenhouse and grown for four weeks.

Glucan lyase activity was re-determined as described above and no significant differences in protein activity were observed.

Glucan lyases produce anhydrofructose from starch. To analyse for differences in the starch content between soil grown wild type plants and transgenic plants accumulating active glucan lyase, leaves were stained with iodine according to Visser et. Al., 1991 Mol. Gen. Genet. 225, 289-296. Leaves from plants grown in the greenhouse with a 16-hour photoperiod were excised from the plants after they had received 8-hour of light. Qualitative starch determination clearly shows that wild type plants and the transgenic line 8.1 (in which no active glucan lyase could be detected) contain high levels of starch. In contrast, no or very little starch could be detected from the transgenic lines 11.1, 14.1 and 14.4. These results suggest that the introduced glucan lyase degrades the starch for the production of anhydrofructose (figure 1).



Wild type (wt) and transgenic potato was stained with Lugol solution (iodine/potassium iodide solution) to qualitative reveal the starch content. Result shows that nostarch could be detected in transgenic lines 11.1 14.1 and 14.3 all containing an active glucan lyase, Glq1, gene. In contrast, both wild ty plants and the transgenic line 8.1 (in which no active glucan lyase could be detected) clearly contain starch.

Figure 1

To determine anhydrofructose accumulation in the transgenic plants neutral and phosphorylated sugars was extracted. Approximately 300 mg of leaf tissue was transferred to 50 ml polypropylene falcon tubes and frozen in liquid nitrogen. After evaporation of the liquid nitrogen 5 ml of 80% EtOH was added and the tubes was placed in a water bath at 80 deg C for 15 minutes. The supernatant was removed to new tubes and the plant material was re-extracted with 5 ml 25% EtOH at 0 C for 30 minutes following a re-extraction with 2 ml water at 0 deg C for 15 minutes. The plant material was washed twice with 2 ml water and all the supernatants were combined. 2 ml of dichlormethane was added and the tubes were mixed gently before centrifugation for 10 minutes at 3000 rpm. The water phase was transferred to new

tubes and the dichlormethane extraction was repeated. The water was evaporated by freeze-drying over night and the final pellet was dissolved in 200 microlitres H_2O.

Anhydrofructose was determined by reacting 200 microlitres total sugar extract with 200 microlitres 3,5 dinitrosalicylic acid for 10 minutes at room temperature before the absorbance at 546 nm of the reaction mixture was determined as described by Yu et. in patent WO94/09122.

Results are shown in Table 2.

Table 2:

Sample	reaction temp C	reaction time	Absorbance 546 nm	Micrograms AF	micrograms AF/g fresh weight
			Before dephosphorylation		
Blank	40	10 min	0		
Wt	40	10 min	0.003	**0**	**0**
line8.1	40	10 min	0.005	**0**	**0**
line 11.1	40	10 min	0.017	**2,2**	**7.1**
line 14.1	40	10 min	0.021	**2.8**	**9.2**
line 14.3	40	10 min	0.023	**3.1**	**10.3**
line 29	40	10 min	0.019	**2.5**	**8.2**
			After dephosphorylation		
Blank	40	10 min	0		
wt	40	10 min	0.009	**0**	**0**
line8.1	40	10 min	0.007	**0**	**0**
line 11.1	40	10 min	0.041	**12.3**	**37.9**
line 14.1	40	10 min	0.048	**14.6**	**45.0**
line 14.3	40	10 min	0.039	**11.9**	**34.9**
line 29	40	10 min	0.033	**9.5**	**28.1**

The results showed that anhydrofructose could neither be detected in wild type potato leaves nor in line 8.1, which does not accumulate detectable glucan lyase activity. However, in line 11.1, 14.1 14.3 and 29 between 9 to 12 micrograms anhydrofructose/g fresh weight could be detected.

Many sugars also exist in phorphorylated forms and since it has been shown that yeast and rat brain hexokinases phosphorylated 1,5-anhydro-D-fructose and its metabolite anhydroglucitol (Taguchi et al., 1993, Biotechnol. Appl. Biochem. 18, 275-83) neutral and phosphorylated sugars was incubated in the presence of calf intestinal alkaline phosphatase (CIAP, Roche) to dephosphorylate all phosphorylated sugars being substrates for the phosphatase. Total sugars was re-extracted from 600 mg leaf tissue and dissolved in 200 microlitres H_2O as described above. 100 microlitres sugar extract was incubated with 50 units CIAP in a reaction volume of 200 microlitres at 37 deg C for 4 hrs in the buffer recommended by the manufacture before anhydrofructose was measured as described above. Results showed that in line 11.1, 14.1 14.3 and 29 between 37 to 54 micrograms anhydrofructose/gr fresh weight could be detected in the dephosphorylated samples.

This demonstrates that transgenic potato plants engineered to accumulate active glucan lyase protein produce anhydrofructose.

A more sensitive method to detect anhydrofructose described by Kametani et. Al., 1996 J. Biochem. 119, 180-185, was used to confirm the above results.

Total sugar extracts was isolated from wild type plants and transgenic line 11.1. One gram of leaf tissue was
homogenized using a Kontes grinder no.21 in 5 ml 150 mM NaCl followed by the addition of 6 ml 40 mM Tris HCl pH 6.0 and 35 mg O-ethylhydroxylamine. Cell debris was removed by centrifugation at 5000 x g for 15 minutes and the supernatants were transferred to new tubes and left at room temperature in the dark for 12 hours for the derivatization of anhydrofructose. The samples were applied onto a reverse-phase column mounted on a computer aided HPLC system. The column was developed with a linear gradient from 100% water to acetonitrile/water (1:1 by volume) in 30 minutes

at a flow rate of 1 ml/min and the elution was monitored by UV absorbance at 207 nm.

As shown in figure 2, anhydrofructose eluting with a retention time of approximately 10 minutes could clearly be detected from extracts of transgenic line 11.1, where as anhydrofructose was absent in extracts of wild type plants. Quantitative determination of anhydrofructose in extracts analyzed by reverse phase HPLC corresponded well with results obtained from the assays using 3,5 dinitrosalicylic acid.

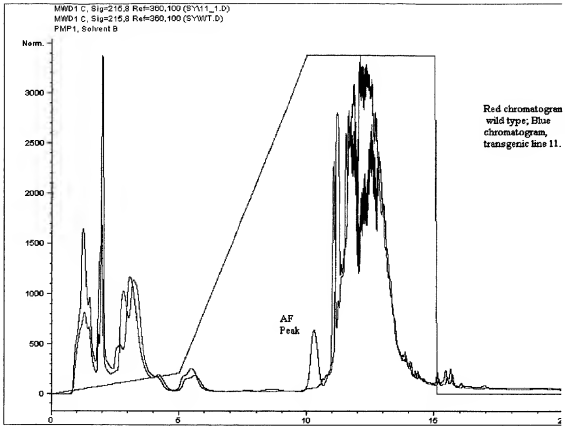

MWD1 C, Sig=215,8 Ref=360,100 (SYW1_1.D)
MWD1 C, Sig=215,8 Ref=360,100 (SYWT.D)
PMP1, Solvent B
Norm.
3000
2500
2000
1500
1000
500
0
0
5
10
15
AF
Peak
Red chromatogram wild type; Blue chromatogram, transgenic line 11.

Figure 2

To test whether other plants also can express an active glucan lyase gene and convert starch into anhydrofructose the same T-DNA construct was introduced into the *Agrobacterium tumefaciens* strain LBA 4404 as described in the patent and used to transform *Arabidopsis thaliana* according to Holtorf et. Al., 1995 Plant Mol. Biol. 29, 637-646.

Glucan lyase activity in Arabidopsis plants

To determine whether transgenic Arabidopsis accumulate a functional glucan lyase gene total protein extracts was prepared from leaves of wild type and transgenic lines SR1 and SR2. Approximately 100 mg of leaf tissue was grinded up in 0,3 ml protein extraction buffer (20 mM Tris pH 7,5, 150 mM NaCl and 1 mM EDTA) and centrifuged for 20 minutes at 15.000 rpm at 4 ^{0}C. The supernatant was transferred to a new tube at kept at 4 ^{0}C. Protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad Laboratories) according to the recommendations of the manufacturer. To detect glucan lyase activity, 250 µg total protein extract was diluted with 100 mM potassium acetate pH 5.5 to 250 µl. Then 250 µl 100 mM potassium acetate pH 5.5 with 10 mg/ml glycogen, was added and the reaction mixture was incubated at 40 ^{0}C for 60 minutes. The reaction was stopped by heating at 100 ^{0}C for 2 minutes before anhydrofructose was determined by 3,5 dinitrosalicylic acid under alkaline conditions as described by Yu et. Al., in WO94/09122

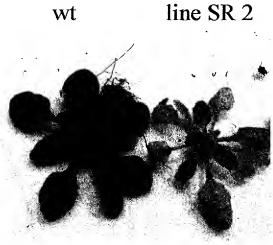
Results showed that lines SR1 and SR2 produced from 82 to 136 µg anhydrofructose per 250 µg total protein extract. No anhydrofructose was observed when using protein extracts from wild type plants.

These results demonstrate that *Arabidopsis thaliana,* similarly to what was observed in potato, can be engineered to express and accumulate glucan lyase activity.

To analyse for differences in the starch content between soil grown wild type plants and transgenic plants accumulating active glucan lyase, leaves were stained with iodine by the same procedure as used for potato.

Qualitative starch determination clearly showed that wild type plants contain high levels of starch. In contrast, no or very little starch could be detected from the transgenic lines SR2. These results show that the introduced glucan lyase degrades starch. As the only product from the degradation of starch by a glucan lyase is anhydrofructose the data shows that anhydrofructose is produced in transgenic Arabidopsis plants expressing a glucan lyase (figure 3).

Starch content in wt and transgenic Arabidopsis thaliane



Wild type (wt) and transgenic Arabidopsis thaliana was stained with Lugol solution (iodine/potassium iodide solution) to qualitative reveal the starch content. Result shows that no Starch could be detected in transgenic line SR 2 containing an active glucan lyase whereas wild type plants clearly contains starch.

Figure 3

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

Signature: Anders Bøgh Jensen

Anders Boegh Jensen

Place:__Frederiksberg___ Date: 2003.11.14